BIOFILM AS A BASIC LIFE FORM OF BACTERIA

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ABSTRACT
Most of bacteria present in the environment grow in the form of biofilms. This structure provides better cells protection against harmful influence of external factors, allows fast and effective intercellular communication and genetic information exchange. Biofilm-forming bacteria may be metabolic differentiated- cells on the surface of this structure have different roperties and carry out other processes than cells in deeper layers. Such intracellular co-operation saves energy and increases the chance of survival.
Biofilms are common in the environment, both natural and anthropogenic. They often develop in water and sewer pipes, on surface of various devices in contact with water and significantly contribute to their destruction. The prevention against biofilm development is very difficult, so researchers still seek new methods of restricting their growth. This goal requires a good knowledge of properties, metabolism and growth rate of this structure, which is gained by conducting various experiments.
The present study reports investigation of *Pseudomonas putida* SM1699 biofilm development in laboratory conditions. This strain has a *gfp* (Green Fluorescent Protein) gene, located in the chromosome. GFP is a protein emitting green light. Application of GFP-labeled strain allows the observation of biofilms using a CLSM microscope (Confocal Laser Skanning Microscope). In this experiment various studies on biofilm, such as number of bacterial cells and the number of cells emitting GFP light were performed. Also the overall number of bacterial cells and the number of cells emitting GFP light were evaluated. The study was conducted in the laboratories of the Silesian University of Technology, as a part of a research grant for gene replacement in the biofilm.

KEYWORDS: biofilm, CLSM, dry weight, CFU enumeration

INTRODUCTION

The bacteria present in the environment often grow in the form of biofilm, called also the biological membranes. This type of structure is very useful and advantageous for bacterial cells, because it allows them to better adapt to changing environmental conditions. Biofilms are communities of single or multiple populations, which are embedded on some type of surface. Bacterial cells included in this structure produce extracellular polymeric substances (EPS), that surround them outside and protect against harmful external factors [18]. The composition of EPS may also include various organic or inorganic ingredients, such as sand or plant remains [7,9].

Biofilms are found in every type of environment, both natural and anthropogenic origin. Their development is conditioned by the presence of water, nutrients and oxygen (for aerobic bacteria). Pathogenic bacteria often form the biofilm in the human body, for example in the lungs, urinary or genital tract. They can grow well on synthetic prostheses, resulting in their destruction. In the natural environment biofilms are present for example in rivers, lakes or soil, as well as in the industrial environment. We often have to deal there with the occurrence of this structure. Bacteria

overgrow water pipes and sewerages, cooling towers, water filters, occur in ventilation equipment and on the surface of various machines in contact with water. Their presence contribute to rapid wear or even destruction of these devices [9,10,12].

Bacteria present in the form of biofilm are more dangerous for several reasons. Firstly, bacteria within biofilms might be very heterogenous, so fighting with them is difficult- agents acting on some bacteria may be ineffective compared to others microorganism. Secondly, dynamic nature of biofilm allows for quick dispersion of large amount of cells and repopulation by new surface. In addition microorganism inside biofilms can communicate effectively with each other via quorum sensing system and regulate various processes [9].

Accurate knowledge of ways of a biofilm forming is very important. In this study *Pseudomonas putida* biofilm was investigated by using different methods for enabling characterization of this structure.

MATERIAL AND METHODS

Bacteria strains and media

Pseudomonas putida SM1699 used in this study was provided by Susanne Koefoed (Laboratory Technician, Technical University of Denmark). This strain has a *gfp* (Green Fluorescent Protein) genes and kanamycin resistance genes, located in the chromosome. It has been described in Table 1.

Table 1. Strain characteristic

Strain	Relevant characteristic	Source
SM1699	*P. putida* R1 (Nal^r) × HB101(RK600) × CC118 λ*pir*(pSM1621); *P. putida* R1 with mini Tn*5*-Km-*rrnB*P1-*gfp*mut3b*-T_0-T_1 cassette randomly inserted into chromosome; Nal^r Km^r	17

This strain was reconstituted from frozen glycerol stock cultures. It was grown in Tryptic Soy Broth (TSB) medium or Tryptic Soy Agar (TSA) medium. If required, kanamycin was added at final concentration 20 µg/ml.

Biofilm growth

Biofilm *P. putida* was grown by two methods. The first method consisted in the cultivation of bacteria in 6-well plates, containing 6 ml TSB each. 100 µl bacteria suspension was introduced into each well and incubated at 37°C. In the second method biofilm was grown in Drip Flow Reactor. Reactor contained 900 ml medium TSB (diluted 1:3 with water), there was introduced 100 ml bacterial suspension. It was incubated at 25°C.

CFU enumeration

The biofilm cell concentration was determined by enumeration of the colony-forming units (CFU). Biofilm was grown on the 6-well plates. Growing area of this plate is 9.15 cm^2. The plates were incubated at 37°C for 24 h. After biofilm formation it was scraped and suspended into 5 ml sterile water.

First method- plate method:

This method measures only viable cells. The results are given as colony-forming units per area of biofilm formation. This method assumes that each colony was grown from a living bacterial cell.

Dilutions to 10^{-10} were made, then were seeded of 10 µl from each dilution on TSA plates, in triplicate. The plates incubated at 37°C for 24 h. After this time the grown colonies were counted. Due to the very rapid growth of bacteria, colonies were counted by dilution of 10^{-10}. The numbers of bacteria were calculated according to the formula:
CFU/ml= (average CFU/drop volume)(dilution counted)
Later it was converted into CFU/ cm^2:
CFU - 1 ml
x - 6 ml
x CFU - 9.15 cm^2
y - 1 cm^2
Second method – McFarland scale:
To determine the CFU was also used the McFarland scale. These standards are often used to evaluate the turbidity of bacterial suspensions and on that basis to determine the number of bacterial cells in the sample. Absorbance of SM1699 suspension was measured at 620 nm and compared with a calibration curve (the blank test- TSB medium) (Tab.2, Fig.1).

Table 2. McFarland standards

Standard No.	Average absorbance (Calibration curve)	No. of bacteria/ml (10^8) represented
0.5	0.111	1.5
1	0.244	3
2	0.523	6
3	0.750	9
4	0.904	12
5	1.157	15
6	1.440	18
7	1.544	21
8	1.705	24
9	1.846	27
10	1.913	30

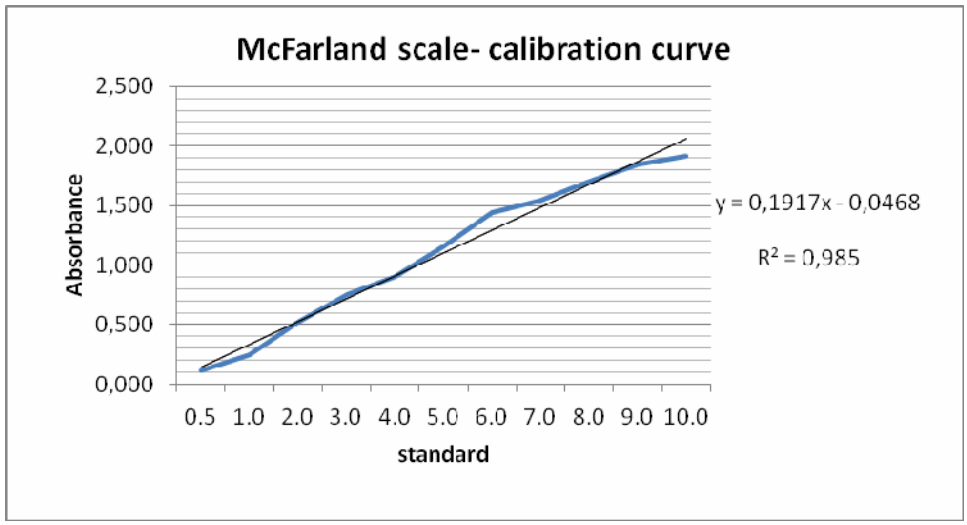

Figure 1. Calibration curve of McFarland scale

Dry weight
The dry weight determination is one of the techniques to calculate the total amount of biofilm biomass. Biofilm growing on 6-well plates for 24 h was scraped into 5 ml of sterile water. Bacteria suspension was filtered through preweighted filter (0.45 µm). Sample was dried in the incubator at 105°C by 2 h and was again weighted. The dry weight of biofilm was calculated based on weight differences.

Crystal violet assay
Crystal violet staining was used to determine the total attached biofilm. Biofilm was grown in 6-well plates at 37°C for 24 h to 72 h. After biofilm formation the medium was removed, then 5 ml of methanol was added. After 15 minutes of incubation methanol was removed, wells were drained and crystal violet was added. Plates were incubated for 5 minutes, next crystal violet was removed. The wells were washed with water twice and acetic acid (33%) was added to each well. The final step was the measurement of absorbance (570 nm). The blank test was acetic acid.

CLSM control of biofilm growth
Biofilm developing in Drip Flow Reactor was observed by using CLSM (Confocal Laser Scanning Microscopy) with Zen Software 2009 Light Edition. To observation was used the EC Epiplan-Neofluar 20x/0.50 HD DIC M27 and lasers 405 nm, 543 nm and 633 nm. The controls were conducted every 24 h for 3 days.

RESULTS

Biofilm grown and CFU enumeration
Both methods of breeding have proved effective. Bacteria multiplied very quickly, which was observed on basis of TSB medium turbidity.
CFU calculation using plate method gave the result $2.1*10^{13}$ CFU/ml, which is equivalent to $1.38*10^{13}$ CFU/cm^2 of biofilm.
In the McFarland scale the obtained result was 1.909. This result was within the range of the 10 standard of McFarland scale and corresponded to $30*10^8$ bacterial cells in 1 ml ($1.97*10^9$ CFU/cm^2).

Dry weight
The weight of dry filter before filtration amounted 0.026 g. The biofilm was filtered through a filter, which was dried at 105°C. After re-weighing it turned out that the weight of the filter with a biofilm was 0.028 g. From the difference between these two values dry weight of bacterial cells was calculated, which amounted to 0.003 g (3 mg).

Crystal violet assay
Crystal violet staining is a rapid method for calculating the biofilm biomass, without its destabilization. The dye first was bound with bacterial cells and later was again resolubilized in acetic acid. The absorbance of final acetic acid solution was measured. The measurement results informed indirect about biomass quantification (Tab.3).

Table 3. Crystal violet staining results (italicised results were rejected)

Wells	24 h	48 h	72 h
I	0.281	***0.119***	0.376
II	0.305	0.39	***0.198***
III	***0.277***	***0.585***	0.412
IV	0.308	0.448	0.288
V	0.245	0.205	0.329
VI	***0.389***	0.562	***0.991***
Average	0.3008	0.3848	0.43233
Average after rejecting extreme results	**0.2848**	**0.4013**	**0.35125**

CLSM control of biofilm growth

SM1699 biofilm development was observed using CLSM microscope. Controls were carried out in I, II and III day since the onset of the culture. Based on the microscopic image it was found that the developing biofilm took on a mushroom structure, with clearly marked microcolonies and water channels. Bacterial cells were covered with a layer of EPS, which made it difficult to distinguish their shape.

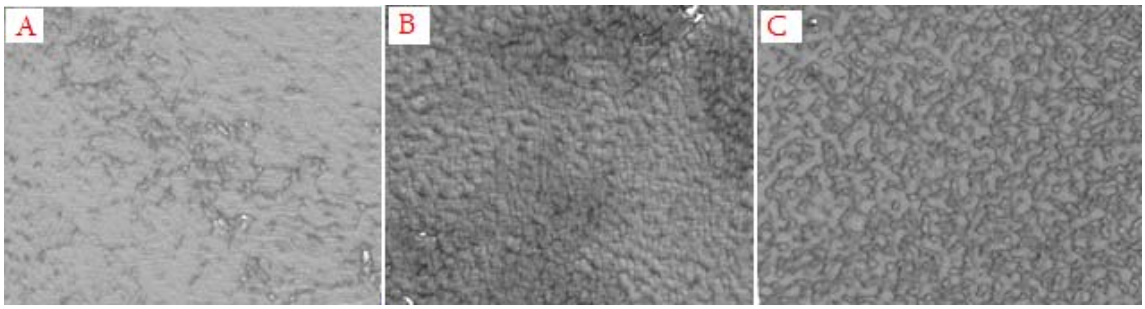

Figure 2. CLSM images of a developing biofilm (3D structure): A- biofilm 24 h; B- biofilm 48 h; C- biofilm 72h

In the first day of culture biofilm resembled a flat, multi-layered structure, covered with a thick layer of EPS, because of which it was difficult to observed individual cells (Fig.2). The depressions corresponding to water channels were visible only in certain areas of the biofilm. In successive days of observations clearly visible was the development of microcolonies (Fig.3), which was dominated in the structure of biofilm on the third day.

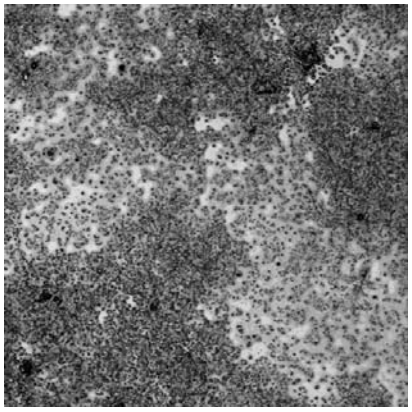

Figure 3. Biofilm of a second day of culture (CLSM images, 2D)

DISCUSSION

Biofilms play an important role in the environment. In the laboratory, you can use several methods of breeding of this structure, in our experiment 6-well plates and Drip Flow Reactor in the breeding were used[16, 20,8].

In our experiment strain *Pseudomonas putida* SM1699 was used, which had mini-transposon in his chromosome, containing the *gfp* and kanamycin resistance genes. This strain was characterized by intense growth, making it possible to fast biofilm development. The study showed that after 24 h of biofilm culture the number of cells was $1.38*10^{13}$ CFU/cm^2. This result indicates a very intense growth of the structure and rapid of cell multiplication in within it. Also was performed an alternative measurement of the number of bacteria in the biofilm, using the McFarland scale [21]. The scale is based on the McFarland turbidity of bacterial suspension in relation to the scale model, drawn from a series of different concentration of barium sulfate solution. Each concentration corresponds to a particular standard in the McFarland scale, and inform about the number of bacterial cells in suspension [21,22].

Conducted research in measuring the number of bacterial cells, made by the method of McFarland showed that in 24 h the suspension is $1.97*10^9$ CFU/cm^2 cells of bacteria in 1 ml. Comparing this result with the result obtained by plate method can be concluded, that this measurement showed a significantly lower amount of bacteria in the biofilm. Probably it is caused by rapid sedimentation of cells, therefore the absorbance measurement is not as reliable as conventional plate method.

CFU measurement is often used to compare the number of bacterial cells present in the form of plankton and biofilm. It is also the basic method of control biofilm development and accumulation of the biomass [1]. These types of measurements were conducted by Lan Liu et al. [13]. They compared the biofilm formation of *Haemophilus influenzae* and *Pseudomonas aeruginosa* in time, using to this purpose the CFU enumeration.

CFU is a measure often used in combination with XTT reduction method, which allows to assess the metabolic activity of biofilm, so you can find a correlation between the intensity of biofilm growth and its activity [15].

Dry weight measurement is a method commonly used to determine the total biomass of biofilm [3,1,4,5]. In this measurement studies it was shown that the biomass of 24-h biofilm was equal to 3 mg. Crystal violet staining is another method for calculating the biofilm biomass, also often used by researchers [13]. This method has one drawback- crystal violet stains not only cells,

but also any material adhering to the surface of the plate (e.g., matrix components); therefore, crystal violet staining may overestimate the number of adherent bacteria [2,5,14].

The results of the studies with use of crystal violet staining indicate, that the biofilm biomass increased in successive days. However, after rejecting extreme results and averaging other results of measurements it can be stated that in the first two days of the observation the biomass of this structure increased whereas in the third day of culture the biomass fell. This may indicate that after 72 h research the bacterial cells began to detach from the walls of the wells, and returned to the form of a suspension, what is one of the final step of biofilm maturation [6,9].

Confocal microscope is a widely used tool for the observation of biofilms because it allows to obtain three-dimensional image of the structure and monitor its development over time without harmful effects on its growth. Visual assessment of the biofilm using a CLSM microscope led to the conclusion, that development of microcolonies clearly occurred only on the third day of culture. Photos taken after the first day showed multilayer, the relatively flat structure, with single water channels, covered with a fairly thick layer of extracellular matrix. In the second day this structure took the form of a more hilly, and after 72 h culture mikrocolonies explicitly protruded above the surrounding surface. Such a differentiation of the biofilm over time is typical for this structure [11,19].

Based on the conducted research can be stated that in the early stages of biofilm growth the biomass is relatively large. Biofilm maturation occurred fairly quickly, as early as third day of culture first signs of aging were observed: a clear differentiation of microcolonies and reduction in total biomass, as demonstrated by crystal violet staining. The conducted studies are part of the larger scientific experiment, designed to illustrate the development of biofilm and phenomena occurring in it.

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